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A PHENOL COEFFICIENT STUDY INVOLVING BACTERIAL PLANT PATHOGENS

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The purpose of this investigation has been to determine the bactericidal efficiency of a number of disinfectants with respect to certain plant pathogens. Nearly all the preparations employed can be readily obtained. Some of them are of recent development; whereas others have been in use for a long time. Occasion was also taken to determine the comparative value of these disinfectants upon a limited number of human and animal pathogens.

In this study 19 different species of bacteria were used, besides a variant of one species. The group of plant pathogens included *Erwinia amylovora, Er. carotovora, Phytomonas tumefaciens, Phy. pruni, Phy. stewarti, Phy. flaccumfaciens, Phy. apii, Phy. phaseoli, Phy. campestre, Phy. michiganensis* (both yellow and white strains), and three other, different, unidentified species isolated, respectively, from sweet clover, decaying cabbage, and sweet corn. Members of the coli group were selected for the human pathogens. These were *Eberthella typhi, Eb. dysenteriae, Salmonella para-typhi,* and *S. schotmuelleri.* One other animal pathogen, *Salmonella pullora,* was also included. All of these organisms grow well upon a nutrient medium containing beef extract and peptone, either in the liquid form or when hardened with agar. They will produce an appreciable clouding of nutrient broth during 18 hours of growth. The toxic action of the disinfectants was limited to the growth interval of 18 to 24 hours. Younger or older cultures were not considered.

HISTORY OF METHODS

Much progress has been made in methods of technique for the standardization of disinfectants since the introduction, in 1881, of the "thread method" by Koch (2) and later the use of small garnets by Krönig and Paul (3). No appreciable advance was made until about 20 years later, when the Rideal-Walker (6, 7) method was proposed; this has served as the basis for the various modifications that have been suggested more recently. The "Lancet method" is very similar to the Rideal-Walker method, the chief variation being the substitution of *Escherichia coli* for *Eberthella typhi* as a test organism. These latter two methods were

the first approach toward a standardization of technique and procedure. A serious objection to both methods is the lack of accuracy in the proportion of culture added to the disinfectant. Rideal-Walker used drops as the unit of transfer of culture to disinfectant; whereas the Lancet commission recommended the use of special spoons and designated the unit of transfer as a spoonful. The impossibility of accurate measurement of such units of transfer presents a serious objection to both of the two methods. Drops from different pipettes vary considerably in size, and no form of spoon or loop approaches the accuracy of a graduated pipette. Inevitable variations arising from such indefinite technique could not help but result in lack of harmony when different investigators employed the same method. In order to meet some of the objections and sources of error of former methods, Anderson and McClintic (1), in 1912, proposed the "Hygienic Laboratory phenol coefficient" method. Much attention was given to the standardization of procedure regarding media, test organism, control of temperature, proportion of culture to disinfectant, inoculation loops, and dilution. A plan was outlined for the testing and standardizing of bactericides, both in the presence of a minimum or maximum quantity of organic material.

The principle involved in both of the methods to which reference has been made was to determine the ratio of the killing power of a known dilution of pure phenol within a definite interval. By this ratio the strength of the killing capacity of an unknown disinfectant was indicated as so many times greater or less than phenol. This ratio is known as the phenol coefficient.

The most recent modification of the Hygienic Laboratory method is the one proposed by Reddish (5). In the Hygienic Laboratory method 0.1 cc. of a culture of the test organism was added to 5 cc. of a known dilution of a disinfectant. Reddish used 0.5 cc. of culture. He varied his calculation of the phenol coefficient by dividing the dilution of the disinfectant which did not kill in 5 minutes, but did in 10 minutes, by the corresponding solution of phenol. He endeavored to simplify the procedure so that consistent results could be obtained with a minimum amount of time and material. Possibly, the most vigorous control factor is the specification which Reddish outlines for the test organism. When *Eberthella typhi* is employed, the culture must be capable of resisting dilutions of phenol from 1-90 to 1-100 for 5 minutes at 20° C. and must be killed within 15 minutes by a dilution of 1-90 or stronger.

All of the methods outlined have been developed for use with human pathogens. Little, if any, attention has been given to the adaptation of any of the methods to bacterial parasites of plants. It may be anticipated, however, that little variation would be necessary when other species of bacteria are considered. We have no precedent to guide us in the selection of certain species or groups of plant pathogens as test organisms to serve the same purpose as *Escherichia coli, Eberthella typhi,* or the Staphylococcus group. It is necessary to determine, first, the relative resistance of a considerable number of different species of bacteria associated with disease in plants to the toxic action of different classes of disinfectants before such a selection can be made. If this can be done within a reasonable degree of certainty, the determination of toxicity coefficients will become very much simplified.

After trials were made with the Rideal-Walker, Hygienic Laboratory, and Reddish methods, a plan was formulated which seemed to be suitable for use with plant parasites.

MEDIA AND CULTURES

Stock cultures were carried upon Bacto nutrient agar slants. This medium, when prepared according to directions, showed a final reaction of pH 6.8. Cultures were transferred once each month and, when sufficient growth had developed after 36 to 48 hours, were stored in an ice box. Transfers were made from the stock cultures once each week to Bacto nutrient broth, pH 6.8. Tubes containing 10 cc. of broth were seeded with a loopful taken from a 3-day-old broth culture. These were incubated from 18 to 24 hours, the plant pathogens at 25° C. and the animal pathogens at 37½° C.

TEMPERATURE

All work was done at a room temperature of 20° C. In a small room heated by a hot water radiator, little difficulty was experienced in maintaining a temperature varying not more than one degree. Cultures incubated at 37½° C. were allowed to stand until a temperature equilibrium had been reached.

APPARATUS

Thoroughly cleansed and sterilized glassware and accurately calibrated pipettes are necessary prerequisites. Platinum wire of 23 gauge with a loop 4 mm. in diameter has become a standard.

DILUTIONS FROM STOCK SOLUTIONS AND STANDARD

All solutions were prepared with sterile distilled water. Increments of dilution vary with the disinfectant tested. With compounds of low toxicity, increments of 5 or 10, or less, are desirable; whereas, in the case of more toxic preparations, higher dilution intervals are necessary, until it is evident that the limit of toxicity is approached. All dilutions were prepared on the day they were to be used, and they were kept free from contamination as rigorously as cultures. Stock solutions prepared in the laboratory were stored in amber-colored bottles in a cool, dark place. All proprietary compounds were kept tightly stoppered in the containers in which they were purchased. The phenol used as a standard must congeal not below 40° C. and must also meet all requirements of the United States Pharmacopoeia.

The colloidal sulfur and the sulfur derivatives were prepared by Dr. O. N. Liming, formerly associated with this Department. Pentathionic acid was prepared from barium pentathionate by removing the barium as sulfate. Quantitative analysis showed the acid to be 96.5 per cent pentathionic, 2.5 per cent tetrathionic, and 1 per cent trithionic.

The colloidal sulfur was prepared in a concentrated paste form by passing SO_2 and H_2S simultaneously into distilled water at 0° C. The stock material contained about 70 per cent sulfur and 30 per cent polythionic acids and water. The dilutions used in the tests were made by adding a weighed amount of the paste to the required amount of sterile water. Detailed description of method of preparations and the analyses are given by Liming in a paper recently submitted for publication in Phytopathology.

In order to make a test of hydrogen sulfide in solution, it was necessary to disregard the standard temperature of 20° C. A saturated solution of hydrogen sulfide in distilled water at the temperature of a freezing mixture of ice and salt was used as a stock solution. Dilutions from this were prepared. The amount of the gas in each dilution was determined by titration with iodine and sodium thiosulfate. The dilution flasks were also kept in a freezing mixture throughout the duration of the test.

A stock solution of ethyl mercury chloride was prepared by dissolving 0.5 gm. of the substance in 400 cc. of distilled water containing 10 cc. of N/10 NaOH. After solution was complete, the volume was made up to 500 cc., thus giving a 1-1000 dilution.

TEST PROCEDURE

It was found convenient to employ a series of seven dilutions of a disinfectant. Accordingly, seven sterile flasks, stoppered with cotton plugs, were labelled to represent the series. A sufficient quantity of a dilution was prepared in each flask so that there would be 5 cc. for each organism to be tested. Test tubes were numbered from 1 to 7 (one to represent each flask) and placed in a rack. Starting with the flask containing the highest dilution, 5 cc. were measured with a volumetric pipette from each flask and put into the corresponding test tube.

The action of a disinfectant was determined after only two time intervals—2½ and 15 minutes. These are considered representative periods. Intervals shorter than 2½ or longer than 15 minutes have no special significance, except in special cases. Furthermore, the slight advantage gained when additional intervals, such as 5, 7½, and 10 minutes, are used does not appear to compensate for the time and material required. By the two-interval plan, three organisms can be tested at one time within 27½ minutes.

Into each tube containing 5 cc. of a dilution of germicide, 0.5 cc. of an 18- to 24-hour-old culture of an organism was introduced. When seven tubes were used, the addition of the culture was made at intervals of 20 seconds, each tube being rotated gently afterward to insure thorough mixing and to avoid the formation of bubbles. At the 2½-minute interval, transfers were made in series, beginning with tube No. 1, to tubes of nutrient broth containing 5 cc. Transfers were again made after 15 minutes from the original dilution tubes to fresh tubes of broth. All tubes were quickly flamed previous to transfer to remove small strands of cotton. These were found to be the greatest source of contamination. All cultures were filtered if they showed any evidence of lumps, pellicle, or heavy sediment.

PERIOD OF INCUBATION

Cultures of plant pathogens were incubated for 3 days at 25° C.; whereas, the animal pathogens were held for 2 days at 37½° C.

CALCULATION OF PHENOL COEFFICIENT

In all the methods used for the determination of the strength of disinfectants, phenol has been designated as a standard. We have followed the same precedent. Possibly other compounds may be considered more suitable in the case of plant pathogens. In any

case, the coefficient of efficiency of any compound can be determined by dividing the dilutions which have been found to stop growth at 2½ and 15 minutes by the corresponding dilutions of phenol. Add the quotients and take the average. The figure obtained represents the toxic efficiency of the substance tested compared to the standard with respect to the organism used. This is the phenol coefficient. The following example outlines the procedure, Table 1.

TABLE 1.—Phenol Coefficient Determination of Metaphen (1-500) for *Phytomonas flaccumfaciens

Substance tested and phenol standard	Dilution	Time culture was exposed to disinfectant (in minutes) 2½	15	Phenol coefficient
Metaphen (1-500)	450	—†	—	$\frac{\frac{500}{100}+\frac{700}{200}}{2}=\frac{5+3.5}{2}=4.25$
	500	—	—	
	550	—†	—	
	600	+	—	
	650	+	—	
	700	+	—	
	750	+	+	
Phenol	80	—	—	
	90	—	—	
	100	—	—	
	110	+	—	
	120	+	—	
	190	+	—	
	200	+	—	
	210	+	+	
	220	+	+	

*Culture—18 hours old. Proportion of culture to disinfectant—0.5 cc. to 5 cc.
†Growth after 3 days is indicated by +; no growth by —.

DISCUSSION OF RESULTS

In Table 2 are given the phenol coefficients which have been determined for 14 different compounds when tested against 13 different species of plant pathogens, considering the white strain of *Phy. michiganensis* as a variant. The figures at the bottom of the table indicate the mean toxicity coefficient of each substance for all organisms. These show that metaphen, mercuric chloride, and ethyl mercury chloride manifested the greatest toxicity in the order named. All of them are mercurial derivatives. In the case of any disinfectant, it is important to bear in mind the actual toxic value of each specific dilution employed. For this reason separate columns are included to indicate the actual toxic value of specific dilutions employed in the cases of metaphen, merthiolate, mercuric chloride, hexylresorcinol, and ethyl mercury chloride. The last column at the right shows the mean resistance of each organism

TABLE 2.—Phenol Coefficient Values for Plant Pathogens

Organism	Mercuric chloride	Mercuric chloride (1–1000)	Iodine tincture U. S. P.	Merthiolate	Merthiolate (1–1000)	Metaphen	Metaphen (1–500)	Mercuro-chrome	Pentathi-onic acid	Hydrogen sulfide
Er. amylovora	1775	1.77	30.8	250.0	0.25	4375	8.75	14.58	6.25	3.7*
Er. carotovora	535	0.53	20.2	150.0	0.15	2625	5.25	10.50	1.12	0.0
Phy. pruni	845	0.84	65.8	200.0	0.20	3416	6.82	18.90	77.50	3.0
Phy. michiganensis (yellow)	2133	2.13	23.6	500.0	0.50	2333	4.66	11.58	11.66	2.1
Phy. michiganensis (white)	2133	2.13	23.6	500.0	0.50	2333	4.66	11.58	.....	2.1
Phy. flaccumfaciens	950	0.95	25.5	750.0	0.75	2125	4.25	5.20	27.50	1.3*
Phy. apii	2350	2.35	13.8	731.2	0.73	1031	2.00	15.87	15.31	1.0*
Phy. phaseoli	525	0.52	21.0	175.0	0.17	1125	2.25	3.00	17.50	0.0
Phy. campestre	1025	1.02	33.2	625.0	0.62	2688	5.37	13.12	24.30	2.8
Phy. stewarti	2218	2.21	17.8	93.7	0.09	1719	3.43	5.93	4.68	0.0
Phy. tumefaciens	2000	2.00	28.1	666.6	0.66	1771	3.54	7.70	11.45	0.0
Bact. sp. A	1792	1.79	13.1	225.0	0.22	1058	2.12	9.16	8.75	0.0
Bact. sp. B	1567	1.56	16.6	250.0	0.25	2166	4.33	7.66	.....	0.0
Bact. sp. C	1774	1.77	19.5	133.3	0.13	1291	2.58	9.16	4.10	0.0
Mean toxicity coefficient for all organisms	1541	1.54	25.18	374.9	0.37	1980	4.28	10.28	17.51	.....

*Calculated for the 15-minute interval only. Growth in all dilutions after 2½ minutes.

TABLE 2.—Phenol Coefficient Values for Plant Pathogens—Continued

Organism	Sulfur (Colloidal)	Zonite	Pepsodent	Listerine	Hexylresorcinol	Hexylresorcinol (1-1000)	Kojene	Ethyl mercury chloride	Ethyl mercury chloride (1-1000)	Mean resistance of each organism for all compounds
Er. amylovora	30.8	3.90	0.16	0.02	125.0	0.12	4.16	1500.0	1.50	451.5
Er. carotovora	4.0	0.17	0.00	0.02	30.0	0.03	4.50	150.0	0.15	196.4
Phy. pruni	275.8	1.76	0.30	0.03	183.3	0.18	5.50	3041.0	3.00	491.0
Phy. michiganensis (yellow)	17.0	0.83	0.15	0.02	91.6	0.09	4.00	2800.0	2.80	440.9
Phy. michiganensis (white)	17.0	0.83	0.15	0.02	91.6	0.09	4.00	2800.0	2.80	466.1
Phy. flaccumfaciens	6.5	0.45	0.20	0.02	75.0	0.07	4.50	2125.0	2.10	339.0
Phy. apii	6.5	1.99	0.19	0.02	19.3	0.01	4.37	2462.0	2.40	369.8
Phy. phaseoli	6.25	0.75	0.20	0.02	100.0	0.10	4.00	525.0	0.52	139.2
Phy. campestre	10.00	1.12	0.25	0.03	112.5	0.11	4.00	1712.0	1.70	449.3
Phy. stewarti	3.10	2.18	0.18	0.02	46.8	0.04	0.00	468.7	0.46	254.7
Phy. tumefaciens	10.20	1.64	0.10	0.03	104.0	0.10	2.50	1770.0	1.70	354.5
Bact. sp. A	5.70	2.25	0.09	0.02	27.5	0.02	0.91	541.6	0.54	217.3
Bact. sp. B	3.50	1.46	0.16	0.02	83.3	0.08	1.30*	900.0	0.90	294.3
Bact. sp. C	5.75	1.85	0.18	0.02	66.6	0.06	0.00	144.0	0.14	191.8
Mean toxicity coefficient for all organisms	25.10	1.51	0.16	0.02	82.6	0.78	3.12	1295.0	1.47	.....

*Calculated for the 15-minute interval only. Growth in all dilutions after 2½ minutes.

against the toxic action of all disinfectants. The greatest resistance was manifested by *Phy. phaseoli, Bact.* sp. C., and *Er. carotovora.* It is evident that *Phy. phaseoli* may be considered as a possible test organism, and yet it will be necessary to make trial of other plant pathogens before this can be determined. It is interesting to note that the action of both strains of *Phy. michiganensis* was the same in all cases. The varying degrees of resistance and susceptibility which different species of bacteria show toward different toxic compounds indicate that the only way to determine the disinfectant value of any substance for a specific organism is by actual trial.

An inspection of Table 3 shows that ethyl mercury chloride was less effective against this class of organisms than merthiolate. Metaphen still ranked high, with mercuric chloride second. Greater resistance was manifested against pentathionic acid and colloidal sulfur by these organisms than by the plant pathogens. The results obtained with hydrogen sulfide may or may not be significant. Possibly other methods would be more suitable for determining the bacterial efficiency of this compound. This sulfur derivative shows definite toxicity for five of the organisms tested after the 2½- and 15-minute interval, under the conditions of the test. The data given for hydrogen sulfide, however, were omitted from all calculations. The high degree of effectiveness of metaphen and merthiolate indicates that these compounds may be useful for seed treatment. Colloidal sulfur exhibits remarkably high efficiency against *Phy. pruni.*

Usually attention is given, in phenol coefficient studies, to one-day-old cultures. In some cases, old cultures of an organism may become more resistant; whereas, in others, they may become less. Remarkable specificity is manifested in the toxicity of certain compounds for certain organisms.

TABLE 3.—Phenol Coefficient Values for Animal Pathogens

Organism	Mercuric chloride	Mercuric chloride (1-1000)	Iodine tincture U. S. P.	Merthiolate	Merthiolate (1-1000)	Metaphen	Metaphen (1-500)	Mercuro-chrome	Pentathi-onic acid	Hydrogen sulfide
erthella typhi	775.0	0.77	18.3	600.0	0.60	1792	3.58	2.66	2.70	0.0
lmonella para-typhi	1052.0	1.05	22.6	104.0	0.10	4010	8.00	6.77	6.35	0.0
lmonella schotmuelleri	635.4	0.63	13.8	36.4	0.03	2344	4.68	5.41	3.54	0.0
erthella dysenteriae	936.8	0.93	25.1	213.7	0.20	1737	3.47	7.99	1.31	0.0
lmonella pullora	1400.0	1.40	23.5	750.0	0.75	2750	5.50	13.83	7.00	0.0
aphylococcus aureus	166.6	0.16	22.3	62.5	0.06	2250	4.50	5.33	3.66	2.3
ean toxicity coefficient for all organisms	827.6	0.82	20.9	181.9	0.29	2480	4.95	6.99	4.09	.....

TABLE 3.—Phenol Coefficient Values for Animal Pathogens—Continued

Organism	Sulfur (colloidal)	Zonite	Pepsodent	Listerine	Hexylresorc-inol	Hexylresorc-inol (1-1000)	Kojene	Ethyl mercury chloride	Ethyl mercury chloride (1-1000)	Mean resistance of each organism for all compounds
erthella typhi	2.91	2.86	0.15	0.02	33.3	0.03	2.66	88.3	0.13	184.50
lmonella para-typhi	5.10	3.20	0.15	0.02	72.9	0.07	1.35	145.8	0.14	330.20
lmonella schotmuelleri	1.56	1.76	0.07	0.02	36.4	0.03	1.45	145.8	0.14	179.50
erthella dysenteriae	3.16	4.10	0.12	0.01	25.6	0.02	2.56	153.7	0.30	173.11
lmonella pullora	1.16	1.73	0.10	0.02	183.3	0.18	1.16	83.3	0.03	301.91
aphylococcus aureus	3.66	1.60	0.09	0.01	66.6	0.06	8.75	233.0	0.15	157.20
ean toxicity coefficient for all organisms	2.92	2.54	0.11	0.01	69.68	0.06	2.98	141.6	0.14	.....

SUMMARY

A simple method is outlined for the determination of phenol coefficients of bactericides, using plant pathogens as test organisms.

Bacto nutrient agar pH 7.3 and Bacto nutrient broth pH 6.8 were adopted as standard media for the purpose of obtaining uniformity of reactions and convenience of preparation.

Using this method, phenol coefficient values were obtained for 14 different disinfectants with respect to 19 different species of bacteria, including 13 plant pathogens and six animal pathogens. These results are presented in tabular form.

The compounds which were found to possess the highest toxic efficiency for the organisms tested are metaphen, mercuric chloride, ethyl mercury chloride, and merthiolate. All of these are mercurial derivatives.

Sulfur derivatives manifested, in general, a greater toxic action upon plant than upon animal pathogens.

Because of the high toxic value of metaphen (chemically known as 4-Nitro-3-Anhydro-hydroxy mercuriortho cresol) and of merthiolate (sodium ethyl mercurithio-salicylate), these compounds may prove useful for seed treatment.

The marked sensitivity of *Phy. pruni* to colloidal sulfur indicates that this form of sulfur should be of value for the control of the "black spot" disease of stone fruits.

One of the most important benefits to be derived from a phenol coefficient investigation is to determine the class of compounds which manifests the greatest toxicity toward an organism.

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